

**OPTIMIZATION OF MEDIUM FORMULATION ON PRODUCTION OF
RECOMBINANT CHITINASE IN *Escherichia coli*.**

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ABSTRACT

Chitinase is enzyme that hydrolyzing chitin to produce N-acetyl-glucosamine. Chitinase can be found in bacteria, fungi, higher plant, insect and some vertebrates. There are a lots of applications of chitinase as the demands of the enzyme is rising high in the market due to usage in pharmaceutical, biopesticides or food industry. For this study, chitinase enzyme was expressed in recombinant bacteria using *Escherichia coli* as a host. The effect of various medium on expression of chitinase in *Escherichia coli* was conducted in this research. Five medium were studied. LB, TB, SB, SOB and 2x YT were screened. LB medium gave the best result of highest chitinase enzyme activity. Each component of LB medium was study to determine the optimize amount of composition to produce the highest enzyme activity. The best range of every composition obtained from the conventional method was 3.0 g/l to 7.0 g/l for sodium chloride, 1.5 g/l to 4.5 g/l for yeast extract and 10 g/l to 14 g/l for tryptone. The optimization was done using response surface methodology (RSM) conducted by software named Design Expert. The optimal medium composition for high soluble recombinant chitinase was determined as 3.63 g/l of sodium chloride, 4.50 g/l of yeast extract and 13.11 g/l of tryptone. From the experimental, the enzyme activity after optimization was achieved 2.291 U/ml compared to predicted response of 2.409 U/ml. This result shows increment of the activity for 82% than before the optimization which is 0.411 U/ml. It shows that the optimization of the medium formulation to improve the expression and production of chitinase was successfully conducted by using RSM.

ABSTRAK

Enzim kitinase menghidrolisis kitin untuk menghasilkan *N-acetyl-glucosamine*. Kitinase boleh ditemui dalam bakteria, kulat, tumbuhan tinggi, serangga dan beberapa haiwan vertebrata. Terdapat banyak aplikasi kitinase sebagai enzim yang mempunyai permintaan tinggi di pasaran kerana penggunaannya di farmasi, biopesticides dan industri makanan. Untuk kajian ini, enzim kitinase diekspreskan dalam rekombinan bakteria *Escherichia coli* yang dijadikan sebagai perumah. Tujuan penyelidikan adalah untuk mengoptimumkan penghasilan rekombinan kitinase. Pengaruh pelbagai media terhadap ekspresi enzim kitinase pada *Escherichia coli* diselidiki. Lima medium telah dikaji iaitu LB, TB, SB, SOB dan 2x YT. LB medium memberikan hasil yang terbaik dengan menghasilkan aktiviti enzim kitinase tertinggi. Setiap komponen medium LB dikaji untuk mendapatkan nilai komposisi yang optimum untuk menghasilkan aktiviti enzim tertinggi. Julat terbaik untuk setiap komposisi yang diperolehi daripada kaedah biasa adalah 3.0 g/l hingga 7.0 g/l untuk natrium klorida, 1.5 g/l hingga 4.5 g/l untuk ekstrak ragi dan 10 g/l hingga 14 g/l untuk trypton. Proses pengoptimuman dilakukan dengan menggunakan kaedah gerak balas permukaan (RSM) yang dijalankan menggunakan perisian Design Expert. Komposisi media yang optimal untuk penghasilan kitinase rekombinan ditentukan sebagai 3.63 g/l natrium klorida, 4.50 g/l ekstrak ragi dan 13.11 g/l trypton. Daripada uji kaji, aktiviti enzim setelah optimasi medium mencapai 2.291 U/ml berbanding nilai anggaran oleh perisian iaitu 2.409 U/ml. Keputusan ini menunjukkan peningkatan aktiviti sebanyak 82% daripada sebelum pengoptimuman yang hanya menghasilkan 0.150 U/ml. Ini menunjukkan bahawa pengoptimuman medium untuk meningkatkan ekspresi dan penghasilan kitinase telah berjaya dijalankan dengan menggunakan RSM.

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LIST OF SYMBOLS/ABBREVIATIONS

ANOVA	-	Analysis of Variance
CCD	-	Central composite design
DNS	-	Di-Nitro Salicylic Acid
g	-	Gram
g/L	-	Gram per litre
hr	-	Hour
IPTG	-	Isopropyl- β -D-thiogalactoside
mg	-	milligram
mg/L	-	milligram per liter
Min	-	minutes
mL	-	mililiter
mM	-	milimolar
OD	-	Optical density
PMSF	-	Phenylmethysulfonylfluoride
R	-	Correlation coefficient
R ²	-	Regression coefficient
RSM	-	Response surface methodology
rpm	-	Round per minute
SSF	-	Solid substrate fermentation
U/mL	-	Unit enzyme activily per mililiter
%	-	percentage
°C	-	degree Celsius
μ L	-	microliter
μ mol	-	micromole

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CHAPTER 1

INTRODUCTION

1.1 Introduction

Chitin, a β -1, 4-linked homopolymer of N-acetylglucosamine (GlcNAc), is the main structural material in the fungal cell walls, insect exoskeletons and crustacean shells. The β -1, 4- glycoside bond in chitin is hydrolyzed by chitinases, that exist in many species, including bacteria, fungi, insects, even plants and human beings in which chitin are not found (Fan *et al.*, 2007).

Chitinase is the enzyme that degrades chitin. Chitin is a linear polymer of N-acetyl glucosamine residues linked by β -1, 4 glycosidic bonds and is a component of fungal cell walls or arthropod integuments. Endochitinase and N-acetylhexosaminidase are two major enzymes of the chitinase system. Chitinolytic enzymes have broad range of applications. Use as biocontrol agents and conversion of waste chitin produced by shellfish processing industry to single cell protein. It also use in cytochemical localization of chitin/chitosan using chitinase–chitosanase–gold complex, fungal protoplast technology and preparation of chitooligosaccharides (Vaidya *et al.*, 2003).

Bacterial chitinases are considered to be important in digestion and utilization of chitin as a carbon source and they are key enzymes for the recycling of chitin in the environment. Thus far, a number of reports have been published on the molecular cloning and nucleotide sequencing of chitinase genes from aerobic bacteria such as *Bacillus circulans*, *Aeromonas* sp., *Alteromonas* sp. and an anaerobic bacterium *Clostridium paraputrificum* (Morimoto *et al.*, 2001).

The emergence of a new biotechnological industry utilizing recombinant DNA technologies for the production of highly specific biomolecules has increased the demand for sophisticated tools such as restriction enzymes, which enable the researchers to cut and paste DNA fragments (Nikerel *et al.*, 2005).

Since the early development of recombinant DNA technology, *E. coli* has been widely used as a host for high-level expression of recombinant proteins. Large-scale productions of valuable proteins in this expression system are usually achieved using a two-stage process. In the first stage, cells are grown to a high cell density under favorable growth conditions in which protein synthesis is kept at minimum via tightly regulated promoter systems. This is followed by a second stage in which high-level expression of the recombinant protein is achieved upon induction (Nikerel *et al.*, 2006).

E. coli grows in both rich complex organic media as well as in salt-based chemically defined media as long as an organic carbon source is provided. Through the type and concentration of ingredients used, cultivation medium composition directly dictates the amount of biomass produced and therefore can dramatically influence the performance of microbial processes (Nikerel *et al.*, 2005).

In recent years, the cloning and expression of the chitinase gene and the construction of recombinant strains with new capacities have been one of the interesting areas of chitinase studies and applications. Chitinase genes have been cloned and characterized from many microorganisms including *Serratia marcescens*, *Alteromonas*, *Enterobacter agglomeron*, *Streptomyces olivaceoviridis*, *Streptomyces iividans*, *Aeromonas caviae*, and *Bacillus circulans*, some of which were either transformed into plants and bacterial strains to increase their ability to control fungal phytopathogens or were highly expressed in *Escherichia coli* cells to enhance the activity of *Bacillus thuringiensis* to control pests (Yong *et al.*, 2006).

Other microorganisms, including bacteria such as *Bacillus lichiniiformis*, *Bacillus pabuli*, *Nocardia orientalis*, *Vibrio alginolyticus*, and many species of fungi such as: *Myrothecium verucaria*, *Stachybotrys elengans*, *Streptomyces cinereoruber*, *Streptomyces lydicus*, *Trichoderma harzianum*, *Trichoderma viride*, *Verticillium lecanii* have a chitinase-producing ability (Liu *et al.*, 2003). Fan *et al.* (2007) also reported that the enzyme also can be expressed in *Escherichia coli* and *Pichia pastoris*.

Response surface methodology (RSM), which is a collection of statistical techniques for designing experiments, building models, evaluating the effects of factors and searching for the optimum conditions have successfully been used in the optimization of bio processes (Nikerel *et al.*, 2005). The concept of RSM has eased process development and has been of significant use at industrial level (Nawani and Kapadnis, 2005).

This RSM method can be used in different ways of optimization process. The method is widely used for optimizing the medium components. Akhir *et al.*, (2009) had successfully optimized medium for chitinase enzyme production from shrimp waste. Vaidya *et al.*, (2003) also had done optimized medium components for the production of chitinase by *Alcaligenes xylosoxydans* culture. Also, the method can be used to optimize culture condition. Pan *et al.*, (2008) has effectively optimized the culture condition enhance *cis*-epoxysuccinate hydrolase production in *Escherichia coli*.

1.2 Problem Statement

Interest in chitinase enzyme application has markedly increased in agricultural industries, but the price is also high due to the complicated process. The switch to chitinase-producing recombinant *E. coli* is seen here as an economic alternative towards higher productivity and easier downstream purification. Growing *E. coli* to high density is currently the method of choice for the production of recombinant proteins, mainly because of the high volumetric productivity associated with this method. Exploring the growth limits of microorganisms in general and *E. coli* in particular, engaged industrial microbiologists many years before it was possible to convert *E. coli* to a “production machine” for heterologous proteins. Thus, optimization of medium for *E. coli* is essential to obtain the highest density of cell growth. Today, techniques to obtain the highest possible density of productive *E. coli* in submerged cultures are well developed.

1.3 Objectives

The main objective in this study is to optimize the production of recombinant chitinase in *E. coli* using Response Surface Methodology (RSM).

1.4 Scopes of Research

The scopes of the study are:

- a) To study the effect of various medium on expression of chitinase in *E. coli*
- b) To study the effect of medium formulation using the best medium
- c) To optimize the medium formulation on production of chitinase in *E. coli* using RSM

1.5 Rationale and Significance

This research will provide the alternative production of chitinase. It helps to increase the production of chitinase while reducing the production cost of chitinase by using the low cost material. The usage of *E. coli* as a raw material in producing chitinase is actually helping reducing the cost as to o. The efficiency of the production is depending on the growth of the *E. coli*. Growing the *E. coli* that contains the recombinant chitinase to high cell density before harvest it will increase the production of chitinase. This research will help to find the optimum medium formulation for the growth of *E. coli* to high cell density.

CHAPTER 2

LITERATURE REVIEW

2.1 Chitinase

Chitinase hydrolyzes the β -1,4-linkage of chitin and is known to be produced by a wide range of organisms, found in bacteria, fungi, higher plant, insect and some vertebrates. Chitin is an insoluble β -1,4-linked polymer of N-acetylglucosamine (GlcNAc) and is one of the most abundant biomasses on earth, second only to cellulosic materials (Morimoto *et al.*, 2001).

Several factors such as chitin, yeast extract, ammonium sulphate, trace elements, tween-20, magnesium sulphate, ammonium chloride, potassium nitrate, diammonium hydrogen phosphate, sodium nitrate, l-glutamine, l-asparagine, peptone and urea have been reported to influence chitinase production by bacteria (Gohel *et al.*, 2006).

The biological functions of chitinases in various organisms are diverse. Plant chitinases were involved in plant defense mechanism against fungal pathogens, however fungal chitinases were associated with autolytic morphogenetic and pathogenesis roles (Fan *et al.*, 2007).

2.1.1 Type of Chitinase

Chitinases can be divided into two categories: exochitinases, demonstrating activity only for the non-reducing end of the chitin chain and endochitinases, which hydrolyse internal β -1,4-glycoside bonds. Many plant endochitinases especially those with a high isoelectric point, exhibit an additional lysozyme or lysozyme-like activity. Chitinases have been reported from several species of plants as two isoforms, acidic and basic. The acidic and basic isoforms of chitinase are induced in plants, in response to pathogen attack and other environmental stimuli and are also expressed in certain tissues of plants during normal development. There are several reports that chitinases isolated from monocot and dicot plants have been shown to inhibit the growth of fungi in vitro (Kirubakaran and Sakthivel, 2007).

The mechanism of hydrolysis of family 18 chitinases has been studied in some detail and these chitinases hydrolyze chitin via a substrate-assisted mechanism, through an oxazoline ion intermediate, involving the carbonyl group of the acetamido group of chitin. Therefore, a single acidic residue is sufficient for catalysis (Kuttiyawong *et al.*, 2008).

A previous study revealed that *Serratia marcescens* produces three distinct chitinase enzymes, ChiA, ChiB and ChiC that favor different modes of catalysis in terms of exo- or endo-type. ChiA and ChiB are processive chitinases that degrade chitin chains in opposite directions, while ChiC is a non-processive endo chitinase. ChiA, a family 18 chitinase, from *S. marcescens* has been extensively studied and exposed aromatic residues on the surface of ChiA has been identified and suggested to be involved in binding and guiding of the substrate into the catalytic cleft during the processive mode of catalysis. Changing these Trp residues to Ala residues reduces the ability of ChiA to bind to its substrate, as well as the activity of the enzyme on insoluble substrates. A proposed model of hydrolysis of chitin by ChiA suggests it is an exo-chitinase hydrolyzing chitin strand from the reducing end (Kuttiyawong *et al.*, 2008).

2.1.2 Classification of Chitinase

Plant chitinases are a diverse group of enzymes with respect to their structure, cellular localization and enzymatic properties. In dicots, the chitinase gene family has been broadly classified into three groups. Class I chitinases are basic and contain a cysteine rich N-terminal domain with putative chitin binding properties. They are usually localized in the vacuole and are potent growth inhibitors in vitro of many fungi. Class II chitinases are generally acidic and extracellular and can be detected in the apoplastic fluid or culture medium of protoplasts. They are not thought to be antifungal either alone or in combination with other proteins. Class III chitinases are extracellular hydrolases whose conserved catalytic domain amino acid sequence differs from the conserved sequence of class I or II chitinases (Kirubakaran and Sakthivel, 2007).

Chitinase belong to the O-glycosyl hydrolases (GH), a widespread group of enzymes that hydrolyze the glycosidic bond between two or more carbohydrates or between a carbohydrate and a non-carbohydrate moiety. A new classification of glycosyl hydrolases based on similarities in amino acid sequences has been put forward. This groups the five known chitinase classes into two GH families, each of which exhibits a strict conservation of the catalytic machinery and enzymatic mechanism. Chitinases of family 18 (formerly class III and V) have been well studied, with information available on the three-dimensional structure and the biochemistry of the enzyme reaction. They operate with retention of the anomeric configuration, whereas family 19 members (class I, II, and IV) invert the configuration at the hydrolysis site. Family 18 of GH (GH18) comprises chitinases from various species, including bacteria, fungi, nematodes, arthropods, mammals and plants, but also several mammalian, insect and plant proteins lacking chitinolytic activity due to substitution of a critical acidic amino acid in the catalytic centre. This new class of proteins has been called chitinase-like proteins (CLPs). Recently, we identified the first lophotrochozoan CLP from the oyster *Crassostrea gigas* (Badariotti *et al.*, 2007).

From previous studies due to amino acid sequence similarity, families 18, 19 and 20 of glycosyl hydrolases can refer to group of chitinolytic enzyme. Chitinase contain from bacteria, fungi, viruses, animals and some plant chitinases are referring to evolutionary term of family 18. While according to family 19, there are consist of plant chitinases (class I, II and IV) (Hart *et al.*, 1995). Both of two families are not share amino acid sequence similarity. However, family 20 is includes the β -N-acetylhexosaminidases from human, bacteria and *Streptomyces* (Dahiya *et al.*, 2006).

2.1.3 Function of Chitinase

In order production of chitinase from organism, there are several function that can be used by organism itself and also industrial sectors. In bacteria, chitinase play roles in nutrition and parasitism while involving morphogenesis in fungi, protozoa and invertebrates. In addition, chitinase are also involved in the defence mechanism of plant and vertebrates (Reetarani *et al.*, 1999).

Furthermore, chitinases are enzyme that capable to hydrolyze chitin to its monomer N-acetyl glucosamine (GlcNAc) (Felse and Panda, 1999). In bacteria chitinase are considered primarily to digest and hydrolyse chitin into a carbon and nitrogen nutrient. On the other hand, chitinase act like catalyst in chitin degradation (Cohen and Chet, 1998).

Fungal chitinase are important for surviving of producing organism itself. This is because they are involved in important morphogenic processes such as spore germination, hyphal elongation and hyphal branching (Kuranda and Robbins, 1991; Takaya *et al.*, 1998).

2.1.4 Chitinase Production

Microbial production of chitinase has captured the worldwide attention of both industrial and scientific environments, not only because of its wide spectrum of applications but also for the lacuna of an effective production method (Nampoothiri *et al.*, 2004).

Production of chitinase is widespread in variety of organisms such as bacteria, fungi, actinomycetes, yeast, plants, protozoans, coelenterates, nematodes, molluscs, arthropods and humans. Several factors such as chitin, yeast extract, ammonium sulphate, trace elements, tween-20, magnesium sulphate, ammonium chloride, potassium nitrate, diammonium hydrogen phosphate, sodium nitrate, L-glutamine, L-asparagine, peptone and urea have been reported to influence chitinase production by bacteria (Gohel *et al.*, 2006).

Solid substrate fermentation (SSF) has recently gained importance for the production of microbial enzymes due to several economic advantages over conventional submerged fermentation. Among the various groups of microorganisms used in SSF, filamentous fungi are most widely exploited because of their ability to grow on complex solid substrates and production of wide range of extracellular enzymes. SSF has been reported to be an economical alternative to submerged fermentation for the production of high titers of proteases and amylases by *Streptomyces rimosus*. Very few reports are available on production of chitinases under SSF conditions, while voluminous literature exists on the production of chitinases by fungi in submerged fermentation (Patidar *et al.*, 2005; Nampoothiri *et al.*, 2004).

In recent years, there has been a surge in the researches regarding chitinase producing *B. thuringiensis* strains: First, the selection of chitinase-producing *B. thuringiensis*. Second, cloning and expression of chitinase-encoding genes of other microbial chitinase in *B. thuringiensis* to enhance the entomotoxicity value. Third, production of endogenous chitinase from *B. thuringiensis* as well as cloning, sequencing of chitinase genes or characteristics of chitinases from some *B. thuringiensis* strains such as *B. thuringiensis* var. *kurstaki*, *B. thuringiensis* var. *parkitani*, *B. thuringiensis* var. *kenyae*, *B. thuringiensis* var. *israelensis*, *B. thuringiensis* var. *alesti* and *B. thuringiensis* var. *sotto* (DangVu *et al.*, 2009).

Morimoto *et al.*, (2001) stated that *C. paraputrificum* is a strictly anaerobic, sporogenic, Gram-positive bacterium. It produces several chitinases when grown with ball-milled chitin as the sole carbon source. Recently, Morimoto *et al.*, (2001) have cloned and sequenced the *chiA* and *chiB* genes encoding the major chitinases in the culture broth of *C. paraputrificum* M21. These chitinase genes form an operon and the domain architectures of ChiA and ChiB are identical, *i.e.*, they comprise a family 18 catalytic domain of glycoside hydrolases, two cadherin-like domains, and a family 12 carbohydrate-binding module in this order.

2.2 *Escherichia Coli* Expression System

Early studies on high cell density growth of aerobic gram-negative bacteria, including *E. coli* were performed either to investigate the limits of bacterial growth in liquid cultures or to obtain large quantities of exponentially grown *E. coli* needed for biochemical studies. During the 1980s, when much information on the genetics and physiology of the bacterium accumulated and *E. coli* became the obvious organism of choice for recombinant protein production, much more emphasis was put on its high-density growth. Since then, numerous methods to obtain high-density cultures have been developed, each aiming at providing means to bypass the physiological constraints that prevent bacteria from growing to the limit of physical barriers between solid state and liquid suspension of cells (Shiloach and Fass, 2005).

Growing *E. coli* to high density is currently the method of choice for the production of recombinant proteins, mainly because of the high volumetric productivity associated with this method. Exploring the growth limits of microorganisms in general and *E. coli* in particular, engaged industrial microbiologists many years before it was possible to convert *E. coli* to a production machine for heterologous proteins (Tokuyasu *et al.*, 1999).

The recombinant expression system in *Escherichia coli* is the most efficient and widely used system for recombinant proteins. With the advent of the post genomic era, the necessity for useful expression systems in *E. coli* has been increasing in order to analyze the functional and three dimensional aspects of proteins encoded by an increasing number of genes. For this purpose, various effective approaches, including different expression vectors and affinity tags such as *Schistosoma japonicum* glutathione S-transferase (GST), His-tag, FLAG-tag, *E. coli* maltosebinding protein (MBP)-tag have been developed (Seto *et al.*, 2008).

Candida antarctica lipase B has previously been expressed in an active form in the *Escherichia coli* cytoplasm and in the periplasm. Eukaryotic expression has been reported in *Pichia pastoris*, *Saccharomyces cerevisiae*, *Aspergillus oryzae* and in *Nicotiana benthamiana* using a viral vector. Prokaryotic expression of active *Candida antarctica* lipase B reported so far has been remarkably lower in terms of yields in comparison to the eukaryotic expression systems. Genetic modifications of *Candida antarctica* lipase B in eukaryotic systems are time consuming in comparison to prokaryotic systems, as are the protein expression and screening of improved *Candida antarctica* lipase B variants in eukaryotic systems. It is therefore of general interest to obtain a wider understanding of the low yield of *Candida antarctica* lipase B in *E. coli* (Larsen *et al.*, 2008).

Studier and colleagues first described the pET expression system, which has been developed for a variety of expression applications. More than 40 different pET plasmids are commercially available. The system includes hybrid promoters, multiple cloning sites for the incorporation of different fusion partners and protease cleavage sites, along with a high number of genetic backgrounds modified for various expression purposes (Sørensen and Mortensen, 2005).

Overexpression of recombinant enzymes in *E. coli* allows the engineering of the enzymes for the study of structure-function relationships by site-directed mutagenesis as well as improvement of the enzymes properties using directed evolution technology. The main limitation of using *E. coli* for secretion of recombinant proteins is that not every protein can be secreted efficiently. Moreover, *E. coli* expression systems are not common for large-scale production of products that are used in food industry because of its potential pathogenicity, only *E. coli* K-12 has been approved for generally recognized as safe (GRAS) status by USA-FDA Food and Drugs Administration (Yamabhai *et al.*, 2008).

2.3 Factor Affecting the Production of Chitinase Enzyme

2.3.1 Effect of Culture Medium on Production of Chitinase Enzyme

The growth of *E. coli* can be limited by other nutritional requirements including carbon, nitrogen, phosphorus, sulfur, magnesium potassium iron, manganese, zinc, copper and some growth factors. When growing *E. coli* to low density, all the required nutrients can be added initially into the basal broth. The popular complex Luria Bertani (LB) broth allows the growth of *E. coli* in a temperature, pH, and oxygen-controlled environment up to a cell density of 1 g/l dcw. To accommodate the nutritional requirements of denser cultures, concentrations of media components must be increased and phosphorus, sulfur and trace elements must be added (Shiloach and Fass, 2005).

Unfortunately, most media ingredients required for growth become inhibitory to *E. coli* when added at high concentrations. It is well established that nutrients such as glucose at a concentration of 50 g/l, ammonium at 3 g/l, iron at 1.15 g/l, magnesium at 8.7 g/l, phosphorus at 10 g/l and zinc at 0.038 g/l inhibit *E. coli* growth. A defined medium that contains the maximum non-inhibitive concentration of nutrients allows growth of *E. coli* to a cell density of about 15 g/l dcw (Shiloach and Fass, 2005).

Escherichia coli is a micro-organism that grows in both rich complex organic media and salt-based chemically defined media as long as carbon source is present. Besides an energy source, it requires nutrients for the biosynthesis of cellular matter, formation of products and maintenance so the content of the medium must supply the nutrients needed to accomplish this. The vital chemical elements needed for the cultivation of *E. coli* are hydrogen, carbon, nitrogen, oxygen, sodium, magnesium, phosphorus, potassium and calcium. These have specific functions during the bioactivity of cell growth and plasmid yield. Physiologically, hydrogen and oxygen form the basis of cellular water and with carbon as the main constituent of organic cell materials. Sodium, potassium, magnesium and calcium are cellular cations and cofactors for some enzymes. Phosphorous constitutes phospholipids, coenzymes and nucleotides in nucleic acids (Danquah and Forde, 2007).

Several factors such as chitin, yeast extract, ammonium sulphate, trace elements, tween-20, magnesium sulphate, ammonium chloride, potassium nitrate, diammonium hydrogen phosphate, sodium nitrate, l-glutamine, l-asparagine, peptone and urea have been reported to influence chitinase production by bacteria (Gohel *et al.*, 2006).

A study by Akhir *et al.*, (2009) use chitin, peptone, yeast extract, sodium nitrate (NaNO_3) and *di*-potassium hydrogen phosphate (K_2HPO_4) as medium for the production of chitinase enzyme from shrimp waste. Vaidya *et al.*, (2003) use ammonium sulfate ($(\text{NH}_4)_2\text{SO}_4$), potassium *di*-hydrogen phosphate (KH_2PO_4), magnesium sulfate~heptahydrate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$), Tween 20, Yeast extract and Chitin for research statistical optimization of medium components for the production of chitinase by *Alcaligenes xylosoxydans*.

2.3.2 Effect of Agitation Rate on Production of Chitinase Enzyme

The major roles of providing agitation in the fermenter are improved mixing, mass and heat transfer. Although increased agitation may provide increased mixing and mass transfer, it may also have many negative effects such as rupture of cells, change in morphological state, decrease in productivity, vacuolation and autolysis. Every fermentation has its own optimum agitator speed. This agitator speed will depend upon the resistance of the organism to shear, its morphological state, the nutrient composition, pH and many other conditions. It has been reported that agitation, pH, dissolved oxygen tension, polymer additives, surface active agents, growth rate and inoculum are the parameters that affect the productivity of a process. Among all these, agitator speed is the most critical parameter and plays a significant role in determining the productivity of the process. Agitator speed becomes important, as it will be one of the most critical parameters used for process scale-up (Felse and Panda, 2000).

Although increase in agitation and aeration may provide better mixing and mass transfer effects, excess in them might result in high shear stress and in turn lead to many negative effects, such as rupture of cells, vacuolation, autolysis and decrease in enzyme productivity. The optimal operation conditions of fermentation process also closely depend upon cell's morphological states, which in turn rely on the resistance of the organism to shear stress. It is generally believed that bacteria could resist more shear stress than fungi, however, little information is provided in the literature (Kao *et al.*, 2006).

2.4 Response Surface Methodology (RSM)

Response surface methodology (RSM) is a combination of mathematical and statistical techniques that is useful for analyzing the effects of several independent variables on the system response without the need of a predetermined relationship between the objective function and the variables. In fact, the relationship between the response and the independent variables is usually unknown in a process; therefore the first step in RSM is to approximate the function (response) through analyzing factors (independent variables). Usually, this process employs a low-order polynomial equation in a pre-determined region of the independent variables. If there is a curvature in the response, then a polynomial of higher degree, such as a second-order model, must be used to approximate the response, which is later analyzed to locate the optimum values of independent variables for the best response value. Using RSM requires special precautions to be taken to determine all critical variables sufficiently as well as not to work with too many variables over wide ranges (Aktasa *et al.*, 2006).

The statistical software package, Design-Expert was used for regression analysis of the experimental data and also to plot the response surface graphs. Analysis of variance (ANOVA) was used to estimate the statistical parameters. To optimize the level of each factor for maximum response, point optimization process was employed. The combination of different optimized parameters, which gave maximum response of the maximum chitinase activity as it was tested experimentally to see the validity of the model (Valappil *et al.*, 2007).

The concept of RSM has eased process development and has been of significant use at industrial level. At a basic biological level, recent studies have indicated the use of RSM for analyzing effects of different factors on proteolytic activity and optimization of xylanase production. This study is an attempt to evaluate the effects of several factors on the production of an industrially important enzyme, chitinase (Nawani and Kapadnis, 2005).